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The RNA helicase DDX1 is involved in restricted HIV-1 Rev function in human astrocytes

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Abstract

Productive infection by human immunodeficiency virus type I (HIV-1) in the central nervous system (CNS) involves mainly macrophages and microglial cells. A frequency of less than 10% of human astrocytes is estimated to be infectable with HIV-1. Nonetheless, this relatively low percentage of infected astrocytes, but associated with a large total number of astrocytic cells in the CNS, makes human astrocytes a critical part in the analyses of potential HIV-1 reservoirs in vivo. Investigations in astrocytic cell lines and primary human fetal astrocytes revealed that limited HIV-1 replication in these cells resulted from low-level viral entry, transcription, viral protein processing, and virion maturation. Of note, a low ratio of unspliced versus spliced HIV-1-specific RNA was also investigated, as Rev appeared to act aberrantly in astrocytes, via loss of nuclear and/or nucleolar localization and diminished Rev-mediated function. Host cellular machinery enabling Rev function has become critical for elucidation of diminished Rev activity, especially for those factors leading to RNA metabolism. We have recently identified a DEAD-box protein, DDX1, as a Rev cellular co-factor and now have explored its potential importance in astrocytes. Cells were infected with HIV-1 pseudotyped with envelope glycoproteins of amphotropic murine leukemia viruses (MLV). Semi-quantitative reverse transcriptase-polymerase chain reactions (RT-PCR) for unspliced, singly-spliced, and multiply-spliced RNA clearly showed a lower ratio of unspliced/singly-spliced over multiply-spliced HIV-1-specific RNA in human astrocytes as compared to Rev-permissive, non-glial control cells. As well, the cellular localization of Rev in astrocytes was cytoplasmically dominant as compared to that of Rev-permissive, non-glial controls. This endogenous level of DDX1 expression in astrocytes was demonstrated directly to lead to a shift of Rev sub-cellular distribution dominance from nuclear and/or nucleolar to cytoplasmic, as input of exogenous DDX1 significantly altered both Rev sub-cellular localization from cytoplasmic to nuclear predominance and concomitantly increased HIV-1 viral production in these human astrocytes. We conclude that altered DDX1 expression in human astrocytes is, at least in part, responsible for the unfavorable cellular microenvironment for Rev function in these CNS-based cells. Thus, these data suggest a molecular mechanism(s) for restricted replication in astrocytes as a potential low-level site of residual HIV-1 in vivo.

Keywords: HIV-1; Rev; DDX1; Astrocytes; DEAD-Box

Introduction

HIV-1 frequently infects the CNS of individuals soon after seroconversion (Resnick et al., 1988; Spector et al., 1993). Studies have demonstrated that microglia and monocyte/macrophages are major cellular reservoirs for highly productive HIV-1 infection in the CNS (Bell et al., 1993; Gabuzda et al., 1986; Gosztonyi et al., 1994; Kure et al., 1990; Resnick et al., 1988). As well, HIV-1 replication has been evaluated in some detail within human fetal and adult astrocytes both in vitro and in vivo (Bagasra et al., 1996; Nuovo et al., 1994; Ranki et al., 1995; Sabri et al., 1999; Saito et al., 1994; Tornatore et al., 1994a). These studies suggest a restricted form of HIV-1 replication in astrocytic cells characterized mainly by expression of multiply-spliced

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HIV-1 mRNA, which encode certain viral regulatory gene products (e.g., Rev, Tat, and especially Nef) (Ranki et al., 1995; Saito et al., 1994; Tornatore et al., 1994a), although this remains controversial (Canki et al., 2001).

HIV-1 infection in astrocytes has several unusual parameters and mechanisms. Human astrocytes have been demonstrated to allow CD4-independent infection with HIV-1, and a recent study has suggested that the human mannose receptor is critical in this entry (Liu et al., 2004), but astrocytes do have functional chemokine receptors (Dorf et al., 2000). HIV-1 expression and transcription may be profoundly different in human astrocytes as compared to human T-lymphocytes (Coyle-Rink et al., 2002; Overholser et al., 2003). Of note, MCP-1, a pro-inflammatory chemokine, may be increased by HIV-1 Tat in human astrocytic glial cells (Abraham et al., 2003), and Tat appears to improve astrocyte survival (Chauhan et al., 2003). Data have also suggested that glutamate transport may be altered in human astrocytes exposed to HIV-1 virions or gp120 (Wang et al., 2003), and subtraction hybridization plus microarray analyses have demonstrated differences in transcriptional regulation in human astrocytes exposed to HIV-1 or isolated gp120 (Galey et al., 2003; Su et al., 2003; Wang et al., 2004).

Persistent HIV-1 infection was initially demonstrated to occur in human fetal astrocytes, which could also be reactivated by various pro-inflammatory cytokines to productive infection, including tumor necrosis factor-alpha (TNF-α) and interleukin-I (IL-I) (Atwood et al., 1994; Tornatore et al., 1991, 1994b). Initial studies suggested that in human astrocytes at least one relative block to highly productive infection was inefficient viral entry (Canki et al., 2001; Kunsch et al., 1989; Liu et al., 2004; Schweighardt and Atwood, 2001). As well, other studies suggested that there might be transcriptional inefficiency of long terminal repeat (LTR)-mediated regulation of viral gene expression (Brack-Werner et al., 1992), as we demonstrated in certain astrocytic cell lines (Niikura et al., 1996). Nonetheless, an over-expression in multiply-spliced viral RNAs, out of proportion to unspliced and singly-spliced viral messages, has been demonstrated by various laboratories in both astrocytic cell lines and primary human astrocytes (Gorry et al., 1999; Kleinschmidt et al., 1994; Kohleisen et al., 1992). This is a pattern consistent with a Rev-negative or more accurately, a Rev-impaired phenotype, which has begun to be more fully characterized in recent studies (Ludwig et al., 1999; Neumann et al., 1995). These findings suggested that this cell type was the first clear example of a dysfunctional Rev response in human cells, leading to an alteration in post-transcriptional control of HIV-1 gene expression (Ludwig et al., 1999).

In a recent article (Neumann et al., 2001), both astrocytic cell lines and primary human fetal astrocytes were evaluated for nucleocytoplasmic transport of HIV-1 Rev. In the vast majority of human cells, Rev is a shuttling protein which is transported from the cytoplasm to the nucleus and returns as the major factor shuttling unspliced HIV-1 RNA from the nucleus of infected cells (Kjems and Askjaer, 2000). These investigators demonstrated that there is a dramatic shift in the level of cytoplasmic as compared to nuclear Rev in astrocytes, with a Rev-deficient viral RNA phenotype characterized by multiply-spliced out of proportion to unspliced viral RNA species (Neumann et al., 2001). In primary astrocytic cells, it was demonstrated that there was a remarkable over-abundance of Rev in the cytoplasm, which did not transport properly into the nucleus, and that nuclear uptake of Rev was impaired specifically. The authors hypothesized that there was a cytoplasmic cellular "activity" that interfered with nuclear uptake of Rev (Neumann et al., 2001). This is important as these are the first initial data on a clear molecular mechanism of HIV-1-impaired production and persistence in a human CNS-based cell type.

Data from Li et al. (2002) have demonstrated that the impaired Rev function in astrocytes may be at least partially alleviated with over-expression of SAM68, the 68-kDa Src-associated protein in mitosis. Of note, though, these studies were performed only in an astrocytic glial cell line, without clear analyses of primary astrocytes, U87.MG.

The differences described in HIV-1 restriction mechanisms in astrocytes, demonstrated in studies from several experienced laboratories, may be based on several potential factors. They include potential multiple levels and mechanisms of HIV-1 restriction in the viral life-cycle in astrocytic cells, difference in stages of cell development, differentiation, or culture conditions used in each study, and/or differences in levels and infectivity of viral input challenges, possibly when utilizing robust viral pseudotypes.

In a high throughput search for cellular factors interacting with the nuclear diffusion inhibitory signal (NIS) motif of the N-terminus of Rev, we identified a DEAD-box (Asp–Glu–Ala–Asp) RNA helicase, DDX1 (Fang et al., 2004). NIS-deficient mutant Rev has a cytoplasmic dominance in Rev-permissive cells, which is similar to the phenotype of intact Rev in astrocytic cells (Fang et al., 2002; Kubota and Pomerantz, 1998; Neumann et al., 2001). We hypothesized that, in astrocytes, alteration of cellular factor(s) interacting with Rev NIS motif leads to an impaired Rev functional status. We demonstrate in this report that DDX1 expression is closely correlated to Rev function in primary astrocytes, with DDX1 over-expression in human astrocytes both normalizing sub-cellular localization of Rev and potently augmenting HIV-1 replication. Therefore, an alteration of DDX1 expression is likely involved in the unique cellular microenvironment of astrocytes.

Results

Ratios of spliced to unspliced HIV-1-specific RNA suggest altered splicing machinery in human astrocytes

HIV-1 inefficiently infects astrocytic cell lines and primary astrocytes, and entry is one clear block towards

HIV-1 replication. Thus, we attempted to bypass the entry block by applying pseudotyped HIV-1 to cultures of primary human astrocytes, as reported previously (Canki et al., 2001).

The purity of human primary fetal astrocytes was confirmed by Cy3-conjugated anti-glial fibrillary acidic protein (GFAP) staining (Fig. 1A). Virus input was optimized to a total amount of 10 ng of p24 Gag at a concentration of 50 ng of p24 antigen per milliliter of culture medium, under which reproducible low-levels of produced virions were demonstrable in the medium of astrocytes, as compared to non-glial HeLa cells (Fig. 1B). With previous studies suggesting the restriction of HIV-1 in astrocytes, we analyzed viral mRNA splicing pattern changes by using a newly-developed semi-quantitative RT-PCR (Fig. 1C). The unspliced *pol* RNA and singly-spliced RNA (BSS-KPNA) representatives in astrocytes were ~30% of that in HeLa cells at a wide range of viral input levels (Fig. 1D, top and bottom panels). In contrast, the multiply-spliced RNA, *tat–rev* and *tat–rev–nef*, demonstrated 30–100% higher relative amounts in human astrocytes over those found in infected HeLa cells (Fig. 1D, 2nd and 3rd panels). We also investigated HIV-1-specific RNAs from day 3 samples and high viral input doses (>1.0 μg/ml, total >200 ng p24 equivalents of virus). In these studies, there was no relative difference found for the various RNA species between astrocytes and HeLa cells (data not illustrated), which suggests that the Rev-based

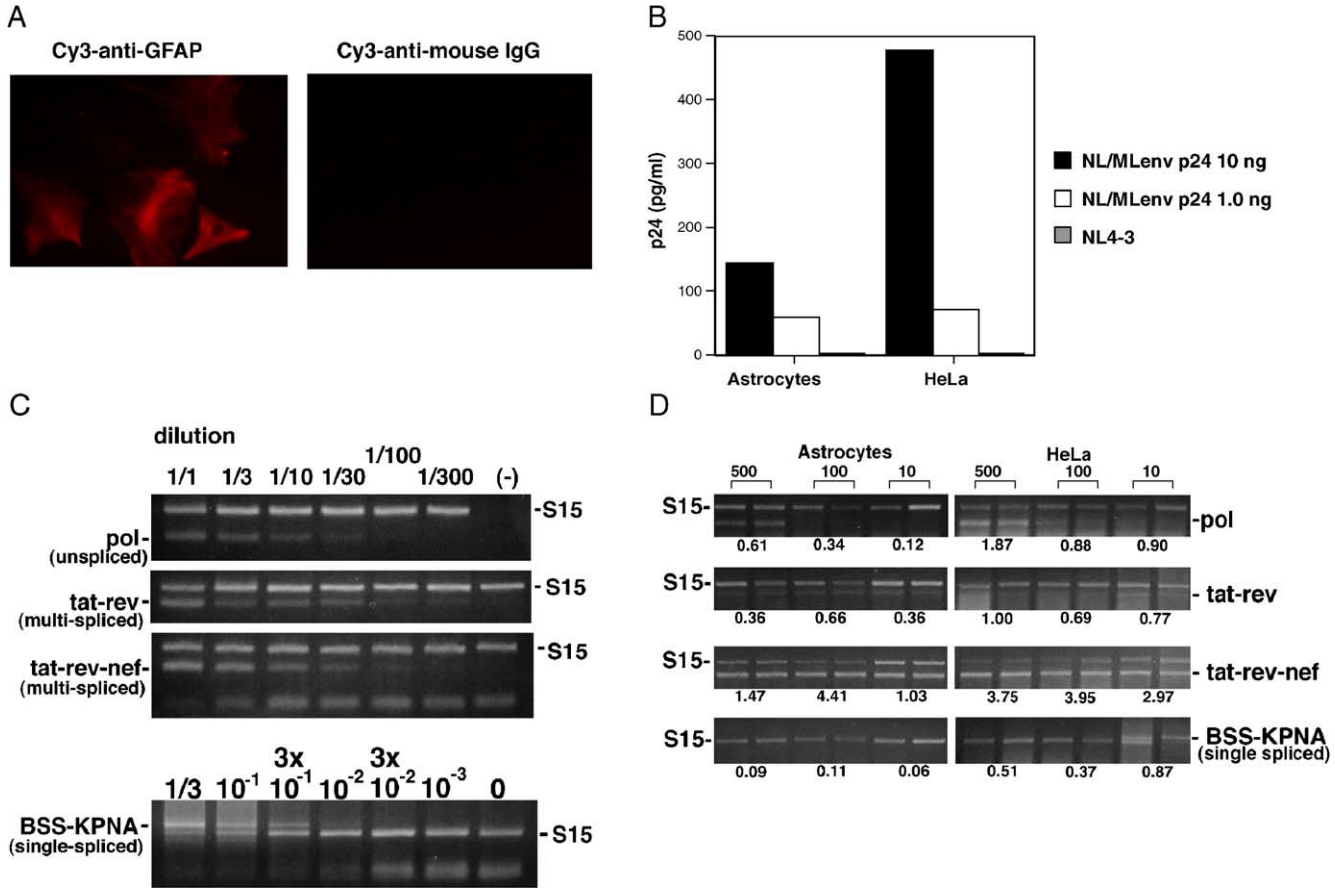

Fig. 1. Accumulation of multiply-spliced HIV-1 mRNA in primary human astrocytes. (A) GFAP expression in human fetal astrocytes. Human fetal astrocytes seeded in slide chambers were stained with Cy3-conjugated, anti-GFAP antibody (left), or with Cy3-anti-mouse IgG (right), as a control. The astrocytes with over 50% confluence, as shown in this figure, were used for infection experiments. (B) Infection by pseudotyped HIV-1_{NL4-3} with murine leukemia virus (MLV) envelope of astrocytes and HeLa cells. For detailed infection procedures, see Materials and methods. The data are representative of two independent experiments. (C) Detection of unspliced HIV-1 genomic RNA, multiply-spliced, and singly-spliced viral RNA in limiting dilution series. The cellular RNA was extracted from the HIV-1_{NL4-3} infected astroglial cell line, U87.MG, followed by reverse transcription and PCR. Mouse cDNA from liver RNA served as a diluent in the limiting dilution series. The control PCR primers, for the 361-bp fragment of "housekeeping" gene S15 of both human and mouse, were combined with primers for amplification of viral RNAs in the same reactions. The sizes for *pol*, *tat–rev*, and *tat–rev–nef* amplicons were 196, 173, and 146 bp, respectively. (D) Unspliced, multiply-spliced, and singly-spliced viral RNA in primary human fetal astrocytes and HeLa cells with infection of pseudotyped or wild-type HIV-1_{NL4-3}. Human fetal astrocytes and HeLa cells in 24-well plates were infected with pseudotyped HIV-1_{NL4-3} with an MLV envelope. Cell samples on the next day after infection were used for detection of *pol*, representative of unspliced HIV-1 genomic RNA, *tat–rev* and *tat–rev–nef* for multiply-spliced viral RNA, and BSS-KPNA for singly-spliced viral RNA (from top to bottom panels), respectively. The numbers under each panel are mean values of density ratios, from duplicates. A, Astrocytes; H, HeLa cells.

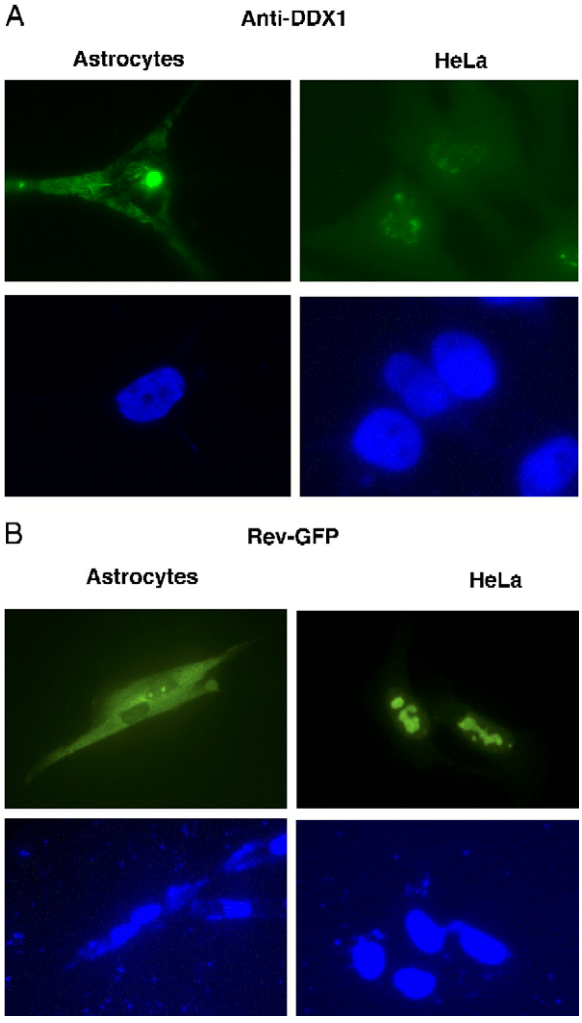

Fig. 2. DDX1 cellular distribution in astrocytes. (A) Endogenous cellular DDX1 in astrocytes (left panel) and HeLa cells (right panel), using indirect immunofluorescence staining and microscopy (see Materials and methods), with DAPI staining (bottom) showing nuclear regions. (B) Expression of Rev-GFP fusion protein in primary human astrocytes and HeLa cells (upper) with DAPI staining (bottom; for details, see Materials and methods).

restriction of HIV-1 in astrocytes is not a fully Rev-negative but rather a Rev-impaired phenotype, which can be evaluated only under certain input virus limits. We conclude that alteration in HIV-1 mRNA splicing machinery occurs in pseudotyped HIV-1_{NL4-3}-infected astrocytes. Thus, cell-type-specific cellular factor(s) may contribute towards post-transcriptional regulation of HIV-1 production in primary astrocytes.

DDX1 is both nuclear and cytoplasmically expressed and HIV-1 rev is cytoplasmically dominant in human astrocytes

As DDX1 was identified as a Rev-interacting protein and Rev was suggested to have cytoplasmic dominance in astrocytes, we first investigated DDX1 sub-cellular distribution in the U87MG astrocytoma cell line, primary human fetal astrocytes, and the non-glial Rev-permissive HeLa cells, as a negative control.

While HeLa cells maintain most DDX1 in the nucleus, both the astrocytic cell line and primary human astrocytes demonstrated some cytoplasmic localization (Fig. 2A). The Rev-green fluorescence protein (GFP) fusion protein in the astrocytic cell line, primary human astrocytes, but not HeLa cells, had mainly cytoplasmic sub-cellular compartmentalization (Fig. 2B). The approximate sub-cellular localizations of DDX1 and HIV-1 Rev in control and experimental groups suggest that cytoplasmic dominance of Rev in astrocytes potentially could be related to DDX1 compartmentalization in these cell types, but distinctive and relative quantitative levels of DDX1 are also likely operational, and associate with aberrant Rev sub-cellular compartmentalization and diminished Rev function in these cells.

Of importance, over-expression of exogenous DDX1 in astrocytic cells induced certain astrocytic cells to demonstrate wild-type nuclear-predominant Rev-GFP localization, while GFP alone remained evenly distributing, including significant cytoplasmic expression (Fig. 3). The reversal of

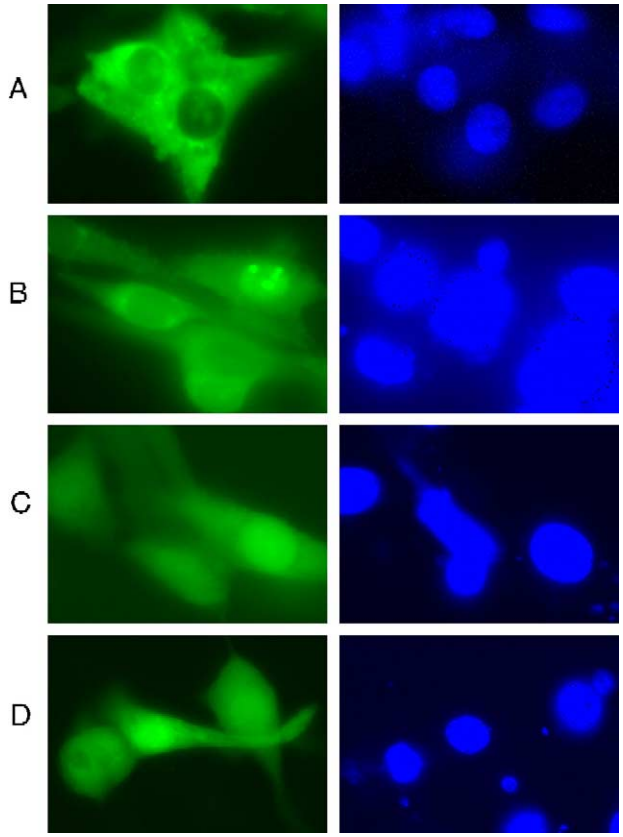

Fig. 3. Additional exogenous DDX1 expression partially restores Rev nuclear localization in astrocytes. Astrocytic cells were transfected with the Rev-GFP expression vector, alone (A) or with pcDDX1 (B). As a control, the GFP expression vector alone (i.e., No Rev fusion; C), or with pcDDX1 (D), was used. Left and right panels show green fluorescence and nuclear DAPI staining, respectively. The total transfection input was normalized with pcDNA.

Rev localization from cytoplasm to nucleus suggests that the basal level of endogenous DDX1 expression, at least relative for astrocytes, may negatively affect Rev function in astrocytes by altering Rev localization.

DDX1-linked alterations of HIV-1 replication in astrocytes

Next, we analyzed if directly altering DDX1 in astrocytes would effect HIV-1 expression. We first showed a specific inhibition of HIV-1 replication by small interfering RNA (siRNA) to DDX1 in astrocytic U87MG cells (Fig. 4A). The efficacy and specificity of siRNA to DDX1 were demonstrated previously (Fang et al., 2004).

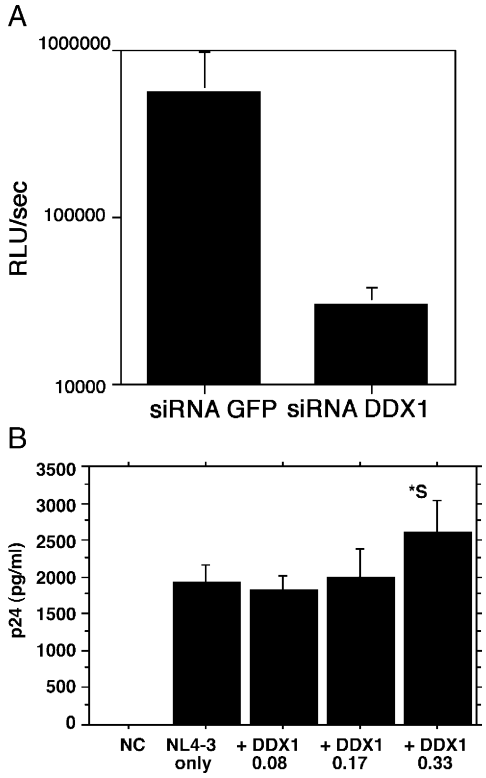

Fig. 4. (A) Further inhibition of HIV-1 replication by down-modulating DDX1 in astrocytic cells. U87CD4/X4 cells seeded in 24-well plates (1×10^5 cells/well) were transfected with siRNA DDX1 or control siRNA GFP at 0.04 μm. Six hours after siRNA transfection, the cells were treated with freshly prepared HXB-*luc* virus (10 ng of p24 antigen) for overnight infection. After washing, the cells were then treated with lysing buffer and luciferase activity was assayed. (B) Augmentation by exogenous DDX1 of HIV-1 replication in primary human astrocytes. Transfections of pNL4-3 alone or with the DDX1 expression vector were performed in primary human astrocytes. The HIV-1 p24 antigen levels (*y*-axis) in the supernatant of cultured cells were measured on the third day after transfection. The numbers in the *x*-axis represent micrograms of DDX1 expression plasmid transfected in each well of a 12-well plate, without (NC, negative control) or with 0.17 μg of pNL4-3 by co-transfection. A total of 0.5 μg DNA, supplemented by pcDNA3.0, was transfected in each well. The data were derived from triplicate experiments with one standard deviation (SD). The symbol "*S" denotes a statistical significance between groups "pNL4-3 only" and "+DDX1 0.33" by using the Student–Newman–Keul method with a significance level of 5% via Statview software.

Approximately 90% inhibition of HIV-1 replication was observed in the siRNA DDX1 group, as compared to the control siRNA green fluorescence protein (GFP) group, demonstrating a potent role for DDX1 in HIV-1 replication in human astrocytic cells. A further confirmation of DDX1's effects on HIV-1 replication in astrocytes was obtained from co-transfection of a DDX1 expression vector with pNL4-3 in primary human astrocytes, demonstrating significantly augmented HIV-1 p24 Gag production in a relatively dose-dependant manner (Fig. 4B). These experiments by-passed low viral entry and directly interdicted in the cellular environment of astrocytes with exogenous DDX1 expression. Therefore, we conclude, based on these data and those illustrated in Fig. 3, that over-expression of DDX1 functions to induce a more favorable intracellular astrocytic microenvironment, which at baseline is semi-restrictive for replication of HIV-1.

Discussion

Wild-type HIV-1 Rev function requires nuclear and/or nucleolar localization of Rev based on a number of reports using mutational and functional approaches (Hope et al., 1992; Kubota and Pomerantz, 1998; Ludwig et al., 1999; Malim et al., 1989). In primary human astrocytes or certain astrocytic cell lines, Rev shifts from nuclear to cytoplasmic dominance, and this was suggested to be one of a multi-step process which leads to limited HIV-1 replication in astrocytes (Brack-Werner et al., 1992; Li et al., 2002; Neumann et al., 1995). These stages include restriction in entry, viral transcription, splicing, Rev-dependant RNA transport, and translation (Brack-Werner, 1999; Gorry et al., 2003; Wang et al., 2004). In this report, we demonstrate that when pseudotyped HIV-1 infection by-pass entry restriction, decreased ratios of unspliced over spliced transcripts developed as an indicator of relative Rev functional inefficiency. A fully functional Rev protein thus requires both critical Rev domains or motifs and specific interacting cellular factors.

The cytoplasmic dominance of Rev distribution in astrocytes was previously shown to retard Rev uptake into the nucleus in a study analyzing dynamic nucleocytoplasmic trafficking of Rev (Neumann et al., 2001). This phenotype of Rev in astrocytic cells, confirmed and extended by our laboratories in the present study, was profoundly similar in terms of cytoplasmic dominance to that of a Rev mutant with deletion of the NIS signal in Rev-permissive cells, as demonstrated in our laboratories (Adachi et al., 1986; Fang et al., 2002). This similarity led us to investigate in astrocytes the NIS-interacting protein DDX1, a DEAD-box RNA helicase for involvement in this altered Rev axis.

The DDX1 gene expresses variably in different cell types (Godbout et al., 1998). While Rev-permissive HeLa cells maintain DDX1 nuclear dominance, specific astrocytic cell

lines and primary human astrocytes reveal both cytoplasmic and nuclear localization of DDX1. Of note, interference with DDX1 expression resulted in aberrant Rev localization, shifting from the nucleus to cytoplasm in Cos-1 cells (Fang et al., 2004). These data, plus the present important functional studies demonstrating that over-expression of DDX1 in astrocytes leads to both Rev sub-cellular redistribution and augmentation of HIV-1 production, support the hypothesis that DDX1 is a critical cell-cofactor for Rev localization and activity in astrocytes.

The precise mechanisms for DDX1's cytoplasmic and nuclear distribution in astrocytes remain to be fully elucidated. Apparently, cellular factors or a related network with DDX1 may be key in "arranging" DDX1 distribution. Secondly, a unique "isoform" of DDX1 expressed in astrocytes may also be possible. The current DDX1 clone used in this present study was derived from a retinoblastoma cell line that frequently shows genomic amplification of DDX1 and the related *N-myc* gene with suggested roles in oncogenesis (Abdelhaleem, 2004; Godbout and Squire, 1993). Of interest, in a recent article, DDX1 has been shown in RNA-transporting granules in dendrites of neurons, together with other related molecules including DDX3, another member of DEAD-box RNA helicase family (Kanai et al., 2004). A very recent study revealed that DDX3 is also required for HIV-1 Rev-RRE nucleocytoplasmic trafficking (Yedavalli et al., 2004), and so synergistic actions of multiple DEAD-box helicases may be co-factors in the lentiviral life cycle. Thus, changes in RNA helicases themselves and/or in the cellular microenvironment of astrocytes will impact on HIV-1 transcript processing.

Finally, the mechanism(s) of aberrant of Rev localization may serve as a molecular key toward understanding unique astrocyte biology. This may also lead to further understanding of potential non-T-cell HIV-1 reservoirs.

Materials and methods

Cells

Primary human fetal astrocytes, only low passage (i.e., less than two), were obtained from Dr. Brian Wigdahl's (Drexel University) and Dr. Avi Nath's laboratories (Johns Hopkins University). Human fetal astrocytes, when confluent to 80%, were split 1:3 or 1:4. Trypsin-EDTA was used for cell detachment. Growth medium included: Ham's F12/DMEM high glucose, fetal calf serum, Hepes 1 M solution, sodium bicarbonate 7.5% w/v, penicillin-streptomycin 1×, and glutamine. Purity was analyzed by Cy3-conjugated antibody staining (Sigma #C-9205) for glial fibrillary acidic protein (GFAP). HEK293T, HEK293, HeLa, human fetal astrocytes, and U87MG (human astroglioma cells) were maintained in DMEM medium with 10% FBS. Jurkat T-cells were cultured in RPMI 1640 with 10% FBS at 37 °C in a 5% CO_2 incubator. All fetal astrocyte studies were approved by the various universities' institutional review boards (IRBs).

Constructs, transfections, and infections

All transfection experiments were performed using FuGENE 6 (Roche #1814443), and followed the manufacture's recommended protocols with minor changes. The infection experiments were performed in a Biosafety Level 3 Laboratory. HIV-1_{NL4-3} virus stocks were generated from HEK293T cells transfected with pNL4-3 (3). The procedures for production of pseudotyped HIV-1 [i.e., HIV-1 with amphotropic murine leukemia virus (MLV) envelope glycoproteins] were the same as described previously (Canki et al., 2001). The cells were infected by either wild-type or envelope-pseudotyped HIV-1 for 2 h. After removal of the infectious medium, the cells were maintained in standard culture medium until being harvested the next day. The supernatants were measured for HIV-1 Gag p24 using an enzyme-linked immunosorbent assay (ELISA; Zeptometrix). Cellular RNA was extracted according to the Trizol reagent protocol (Invitrogen #15596-026) and processed with a reverse transcription reaction (M-MLV RT. Promega #BP3208-1). Green fluorescence protein (GFP) and GFP-Rev fusion constructs were as described (Neumann et al., 2001).

HIV-1 proviral clones, expressing the luciferase (*luc*) gene in the *nef* open reading frame of HXB2 (X4-tropic), were used to produce viral stocks after transfection into U87MG cells (Tokunaga et al., 2001). Normalized quantities of virus were used to infect U87MG astrocytic-glial cells constitutively expressing CD4 and CXCR4 on the surface. Synthetic siRNA duplexes were designed and then chemically synthesized by Dharmacon Inc. (Lafayette, CO). The target sites for the siRNA DDX1 and siRNA GFP were CAAGCCCTCTTTCCTGCCTG (961–980, X70649) and GCAGCACGACTTCTTCAAG (918–936, U55762), respectively (Fang et al., 2004).

Primers and PCR conditions

The sizes for unspliced *pol*, multiply-spliced *tat–rev*, *tat–rev–nef*, and singly-spliced RNA (BSS KPNA) were 196, 173, 146, and ~400 bp, respectively. The primers used in the RT-PCR were SCaf.F (5′AAGCTCTATTAGATACAGGAGCAGATG A3′)/Scaf.R(5′CCAATTATGTTGACAG GTGTAGGTCCTA3′) for *pol*, SC02F (5′AGCCTTAGGCATCTCCTATGGCAGGAA3′)/SC52R (5′TAAGTCTCTCAAGCGGTGGTAGCTGAA 3′) for *tat–rev*, SC05F (5′GAAGAAGCGGAGAC AGCGACGAAGAGCTC3′)/SC52R for *tat–rev–nef*, and, BSS (5′ GGCTTGCTGAAGCGCGCA CGGCAAGAGG3′)/KPNA(5′AGAGTGGTGGTTGCTTCCTTCCACACAG3′) for singly-spliced 4.0

kb mRNA (Bilodeau et al., 2001; Brachtel et al., 2002; Schwartz et al., 1990). The internal control PCR primers, for the 361-bp fragment of the "housekeeping" gene rig/S15, were S15-F (5′ TTCCGCAAGTTCACCTACC3′) and S15-R (5′ CGGGCCGGCC ATGCTTTACG3) (Inoue et al., 1987; Kitagawa et al., 1991). PCR mixtures were prepared by adding 1.0 μl/20 μl of RT reaction to a total of 20 μl PCR buffer (10 mM Tris–CL pH8.3, 50 mM KCL, 0.2 mM each dNTP, 4.0 mM MgCL2, 0.01% Gelatin and 0.05% Tween 20 with 0.04 μM of each housekeeping gene mRNA primer, 0.2 μM of each target primer, 0.4 Units of Taq polymerase; Fisher Scientific). Two thermal cycle programs were used: 94 °C 5′/30 cycles of 94 °C45″–59 °C45″–72 °C60″/72 °C7′ for unspliced/multiply-spliced, and 94 °C 5′/40 cycles of 94 °C45″–61 °C45″–72 °C60″/72 °C 7′ for singly-spliced RNA, with GeneAmp PCR system 9700 (PE Applied Biosystem). Ten microliters of each PCR product was applied to a 2% agarose gel for electrophoresis and than visualized with ethidium bromide staining.

Semi-quantitative assays of RT-PCR products

The gel photographs were scanned and further analyzed for density comparison by using IQ Mac v1.2 software (Molecular Dynamics). The density ratios of viral RNA bands versus the "housekeeping" gene band served as standards, according to the known dilution of the samples in cDNA from HeLa cells.

A mouse anti-DDX1 monoclonal antibody (BD Pharmingen) was used at a dilution of 1:200 in immunofluorescence analyses (IFA). The fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma) was employed as a second antibody. The cells cultured on chamber slides were fixed 48 h after transfection with 3.5% formaldehyde in Dulbecco's phosphate-buffered saline (PBS). Incubation in primary and secondary antibodies was performed for 1 h at 37 °C, followed by extensive washes with PBS. The cells were then treated with mounting medium containing 4′, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Cells expressing GFP were processed through similar washing, fixation, and mounting steps. The fluorescence from these cells was analyzed via epifluorescence microscopy (Olympus).

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